# PHYSICAL AND CHEMICAL COMPARISONS OF UNIVALENT AND MULTIVALENT SEA URCHIN FERTILIZINS [1]

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Solutions of most sea urchin egg jellies can agglutinate the sperm of the species (Lillie, 1913; Tyler, 1940; Vasseur, 1951; for most recent review see Monroy, 1965). This reaction is usually species-specific (Tyler, 1949) and is believed to be analogous to antibody agglutination of cells. The sperm agglutinin, called fertilizin, is the principal component of the egg jelly (Tyler, 1941).

In keeping with immunological doctrine, Tyler (1948) proposed that agglutinating fertilizin must be multivalent in terms of the combining groups (active sites) on the molecule. Tyler (1941, 1942, 1948) and Metz (1942, 1954) showed that fertilizin could be rendered non-agglutinating (univalent) without destroying its capacity to combine with the specific complementary sites of the sperm.

The chemistry of various multivalent sea urchin fertilizins has been studied. Tyler and Fox (1940) obtained a positive biuret test with *Strongylocentrotus purpuratus* fertilizin. This and the fact that proteolytic enzymes destroyed the sperm-agglutinating properties of their preparations, was interpreted to mean that the fertilizin was of a protein nature. Vasseur (1948) showed that the fertilizins of *Paracentrotus lividus, Echinus esculentus, Stronglyocentrotus droebachiensis,* and *Echinocardium cordatum* contained nine different amino acids. The nitrogen content of these different fertilizins varied from 2% to 5% of the dry weight. Sulfur and sulfate analyses indicated that the materials contained roughly 25% to 30% sulfate. Vasseur (1952), in summary of these and other findings, concluded that fertilizins in general are 20% to 25% protein and 75% to 80% carbohydrate, with sulfate groups esterified to the carbohydrate. Monroy (1965) reviews the chemical compositions of several fertilizins.

Sulfates esterified to carbohydrates would tend to give the fertilizin molecule a negative charge at neutral pH. The negative charge of the molecule was confirmed by Runnström *et al.* (1942) and Tyler (1949, 1956) who demonstrated that fertilizin migrates during Tiselius electrophoresis as a single component with high anodic velocity. The mobility changes very little over the pH range of 3 to 9.

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The sedimentation behavior (Runnström *et al.*, 1942; Tyler, 1956) shows that the molecule is a high molecular weight, asymmetric particle. The sedimentation velocity increases with decreasing concentration for *Psammechinus miliaris* (Runnström *et al.*, 1942) and *Arbacia punctulata* (Tyler, 1956) fertilizins.

The relationship of univalent to the multivalent fertilizins has not been studied seriously by chemical and physical techniques. Tyler (1941), and Metz (1957) hypothesized that the conversion to the univalent form involves a fragmentation of the fertilizin molecule. Fragments containing the active sites for combination with sperm must be relatively large since they fail to pass through dialysis membranes (Tyler, 1941; also see Krauss, 1949). The only direct evidence for fragmentation is found in experiments of Hathaway and Metz (1961) who demonstrated release of an inert fertilizin component(s) following spontaneous reversal of agglutination. These workers, however, did not establish whether fragmentation is a uniform process resulting in one or several subunits, comparable to antibody fragmentation to a univalent form (Porter, 1959); or whether cleavage of the molecule is random, involving formation of a spectrum of fragments; or whether it simply involves a progressive destruction of active sites leaving only one in the final univalent material (Tyler, 1941).

The present work describes some of the physical and chemical properties of univalent *Lytechinus variegatus* fertilizin and includes comparison with those of the multivalent form.

## Materials and Methods

### 1. *Preparation of fertilizin*

*Lytechinus variegatus* was collected at Alligator Harbor, Franklin County; Biscayne Bay, Dade County; and Key Largo, Monroe County, Florida. Gametes were obtained either by injecting the animals with isotonic KCl or by cutting the test, which caused shedding of the gametes. Semen was collected without added sea water in Syracuse dishes. Eggs were collected in Marine Biological Laboratory artificial sea water (Formulae and Methods, IV, 1956) and washed three times with this sea water. To prepare egg jelly solutions, the washed eggs were resuspended in artificial sea water, pH 4.5 to 4.9, to a final concentration of approximately 20% by volume. Only those samples which were free from cytolyzed eggs upon microscopic examination were used for further study. After the eggs settled, the supernatant was decanted and centrifuged in a hand-driven centrifuge. The pH was adjusted to 7.5 with NaOH or $NaHCO_3$, and the supernatant solution was centrifuged at 25,000 *g* for 30 minutes at 4° C. to remove any particles. Egg jelly (fertilizin) was then precipitated with a final concentration of 65% ethanol below 0° C. The precipitate was suspended in distilled water, and the suspension dialyzed in the cold against several changes of distilled water, for a maximum time of 72 hours. The undissolved material was removed by centrifuging at 30,000 *g* for 30 minutes at 4° C. See Figure 1 for flow sheet of preparation. The preparations were stored either frozen or at 4° C. Fertilizin retained its sperm-agglutinating capacity for at least one month at 4° C. and at least one year when frozen.

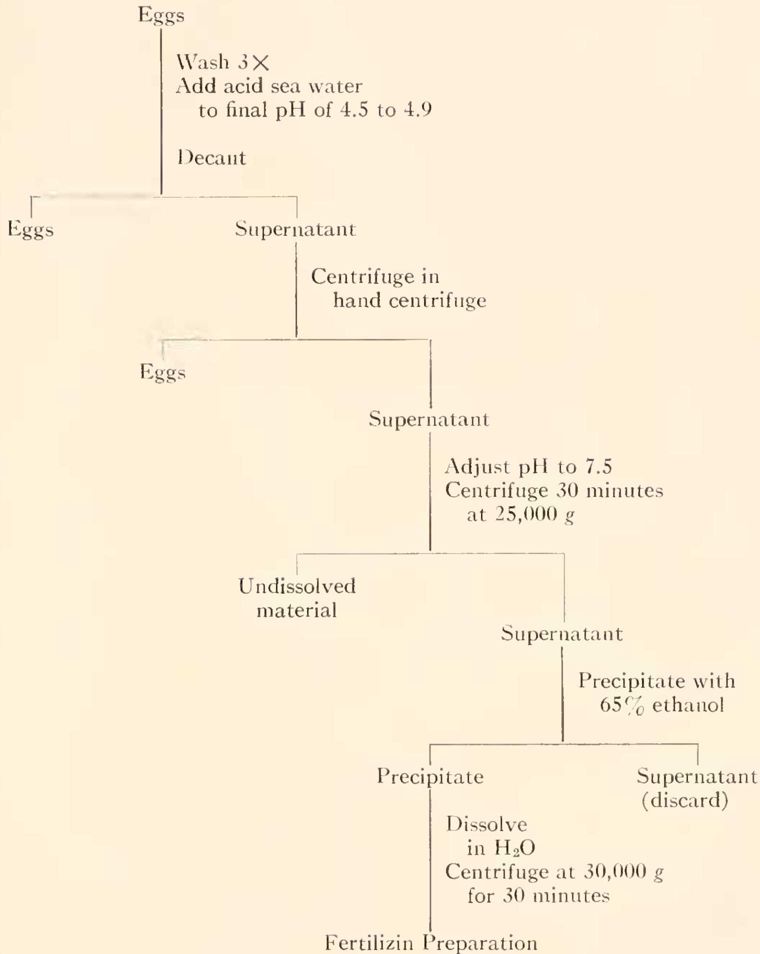


FIGURE 1. Isolation procedure of fertilizin.

## 2. *Preparation of univalent fertilizin*

Fertilizin was converted to the univalent form by a 30-minute treatment with 2% hydrogen peroxide at room temperature (Metz, 1954), followed by dialysis against distilled water or other solvent. Control multivalent fertilizin solutions were treated identically except that water was substituted for hydrogen peroxide.

Operationally multivalent fertilizin samples were defined as those which could agglutinate homologous sperm. A sample was considered univalent when it failed to agglutinate homologous sperm, and when, in addition, these treated sperm could not subsequently be agglutinated by a sample of multivalent fertilizin. Agglutination tests were made by mixing equal volumes of 1% sperm suspensions and the test sample in a Syracuse dish. A control of sperm mixed with sea water was always included.

Most comparisons were made between univalent fertilizin formed by hydrogen peroxide treatment and the parent multivalent material. Univalent fertilizin was also obtained by two other techniques: (1) a spontaneous breakdown of multivalent fertilizin, at times enhanced by an elevation of temperature, and (2) treatment with proteolytic enzymes: (trypsin [Worthington, crystallized], chymotrypsin [C. F. Boehring and Soehne], and pronase [Cal. Biochem., B grade]). Trypsin and chymotrypsin were buffered with 1*M* pyridine:collidine buffer, pH 8.0. Pronase was buffered with 1 *M* pyridine:collidine buffer, pH 8.0, containing 0.02 *M* $CaCl_2$. This volatile buffer was used to avoid salt effect which could interfere with electrophoretic and chromatographic analyses of the hydrolysates.

3. *Physical analyses*

a. Viscosity. Viscosities were determined in a Cannon-Ubbelohde semi-micro dilution viscometer, and an Ostwald-Cannon-Fenske number 200 viscometer with temperature controlled to $\pm 0.1°$ C.

b. Sedimentation velocities. Sedimentation velocity values were determined in a Spinco Model E ultracentrifuge at a speed of 47,660 rpm. Usually two samples were run at a time, using one normal and one wedge cell. The solvent was 0.1 *M* NaCl. Schlieren optics were employed to detect the position of the boundary. Peak displacement was measured on a Nikon Profile Projector at a magnification of 10 × or 20 ×. Sedimentation values were calculated from the slope of the line obtained by plotting 2.3 log x against time (Schachman, 1959). All values were corrected to standard conditions of water as a solvent at 20° C. by the approximation

$$s_{20,w} = s \frac{\eta_t}{\eta_{H_2O}} \text{ (Steiner, 1965).}$$

Solvent viscosity values were obtained from the International Critical Tables (1926) and Lange (1956).

c. Electrophoresis. Electrophoresis on cellulose acetate (Sepraphore III, Gelman Instrument Company) was carried out utilizing barbital buffer, pH 8.6; borate buffer, pH 8.0 and 8.6; and phosphate buffer, pH 6.5, 7.5, and 8.0, $\Gamma/2 = 0.05$. Metachromatic staining in all procedures was with 0.01% Toluidine Blue O in distilled water. Protein staining was attempted with Amido Schwarz or Ponceau S. Samples were destained with 2% acetic acid or 0.001 N HCl. Photography of the stained cellulose acetate strips was performed by making contact negatives of the strips on Kodak Contrast Process ortho film.

4. *Chemical analyses*

a. Carbohydrate analyses. Material for carbohydrate analysis was hydrolyzed in sealed tubes with 1 N HCl at 100 ± 2° C. for from 1 to 24 hours. Excess acid was removed by drying *in vacuo* in the presence of NaOH and $H_2SO_4$ or $P_2O_5$. Subsequently, the material was dissolved in distilled water and dried again. After the second drying, the hydrolysate was dissolved in a minimal amount of water and aliquots were applied to Whatman number 1 or 3 MM filter papers for ascend-

ing chromatography. Solvent systems used were 1-butanol:ethanol:acetone:water, 5:4:3:2 (Gray and Fraenkel, 1954) and 1-butanol:pyridine:water, 54:25:45 (Block *et al.,* 1958). Spots were developed with ammoniacal silver nitrate or aniline-phosphoric acid (Block *et al.,* 1958). Chromatographic comparisons were made with arabinose, glucose, galactose, fructose, rhamnose, fucose, glucosamine, and galactosamine (all Nutritional Biochemical Corporation) standards. Rhamnose and fucose from Cal. Biochem. were also used. Fucose determinations were

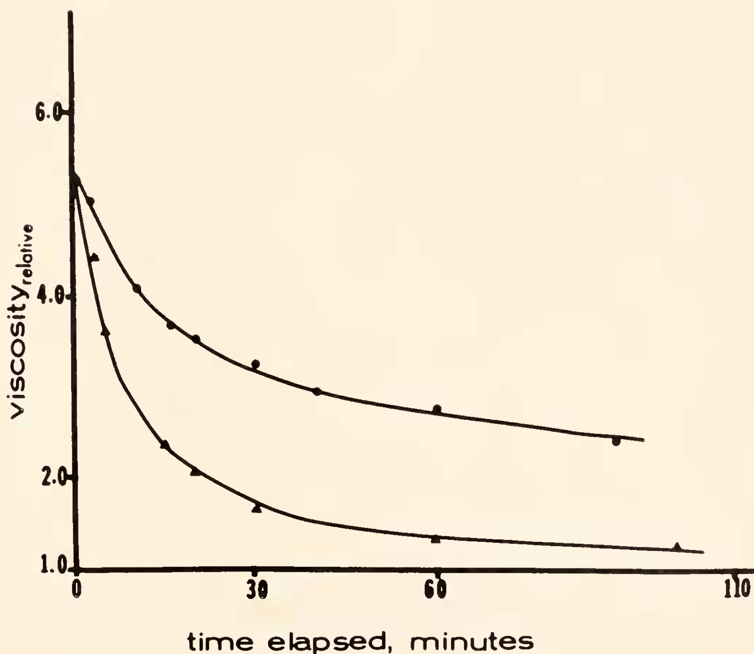


FIGURE 2. Viscosity change of fertilizin in two concentrations of $H_2O_2$. Fertilizin concentration 0.6 mg./ml. Upper curve: 0.4% $H_2O_2$. Lower curve: 2.0% $H_2O_2$. The viscosity of a 5-ml. sample was determined in two Ostwald-Cannon-Fenske viscometers at 30° C. Thirty per cent $H_2O_2$ was added to one viscometer to attain a 2% or 0.4% concentration. An equivalent amount of water was introduced into the other viscometer. The viscosities of the experimental and control solutions were determined at intervals. No change was noted in the control solution in 100 minutes.

done by the CyR3 method of Dische and Shettles (1948). Optical densities were determined in a Beckman DK II spectrophotometer, a Bausch and Lomb Spectronic 20, or a Zeiss PM Q II spectrophotometer.

b. Amino acid analyses. Amino acid contents of univalent and multivalent fertilizins were determined with a Phoenix Model K-5000 automatic amino acid analyzer employing Beckman or Phoenix resins. For the analysis, material was hydrolyzed, with 6 N HCl in sealed evacuated tubes at 110 ± 2° C. for 24 hours or by treatment with the enzyme pronase.

c. Nitrogen. Nitrogen was determined by the Kjeldahl procedure (Fischer, 1961).

Table I

*Sedimentation velocities of multivalent and univalent Lytechinus fertilizins*

| Sample | Concentration g. fucose/ml. | | $s_{20,w} \times 10^{13}$ | |
|---|---|---|---|---|
| | m | u | m | u |
| 8/31, 4 | 195 | 750* | 6.15 | 2.78 |
| 8/31, 3 | 175 | 700* | 5.83 | 2.87 |
| 5/12, 2 | 260 | — | 5.76 | — |
| 5/12, 1 | 300 | — | 5.05 | — |
| 9/1 | 400 | 1025* | 4.48 | 2.78 |
| 9/4, 1 | 750* | — | 3.18 | — |
| 10/1 | — | 220 | — | 2.96 |

* Conc. with Aquacide.
m: Multivalent fertilizin.
u: Univalent fertilizin.

d. Amino sugars. Glucosamine and galactosamine were determined on the Phoenix amino acid analyzer employing Beckman resins. Samples were hydrolyzed with 2 N HCl for 1, 2, 5, and 10 hours in sealed tubes at 105 ± 2° C. Dry weights of samples were determined on a Mettler balance of material dried by lyophilization. Several samples of multivalent and univalent *Lytechinus* fertilizins were concentrated by dialysis against Aquacide (Cal. Biochem.).

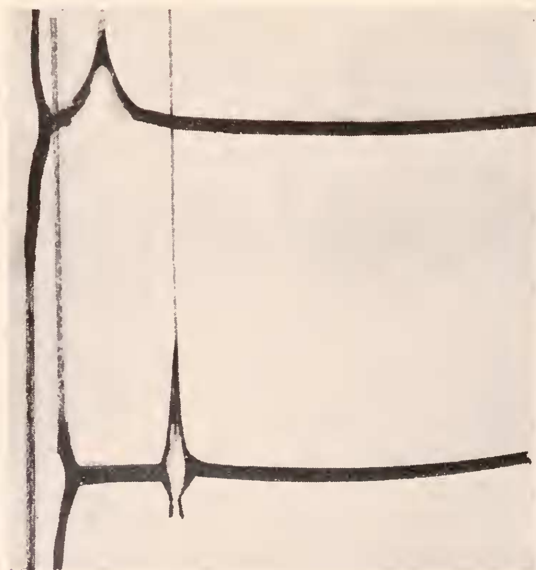

Figure 3. Sedimentation pattern of multivalent and hydrogen peroxide formed univalent fertilizins. Speed was 47,660 rpm at 10° C. Time illustrated is 64 minutes after speed was attained. Bar angle of 45°. Upper pattern: Sample 8/31, 4 U. Lower pattern: Sample 8/31, 4 M.

## RESULTS

### *Viscosity*

Viscosities of four univalent and parent multivalent preparations were compared. In all cases the conversion ($H_2O_2$ treatment) to the univalent form resulted in a marked drop in relative viscosity.

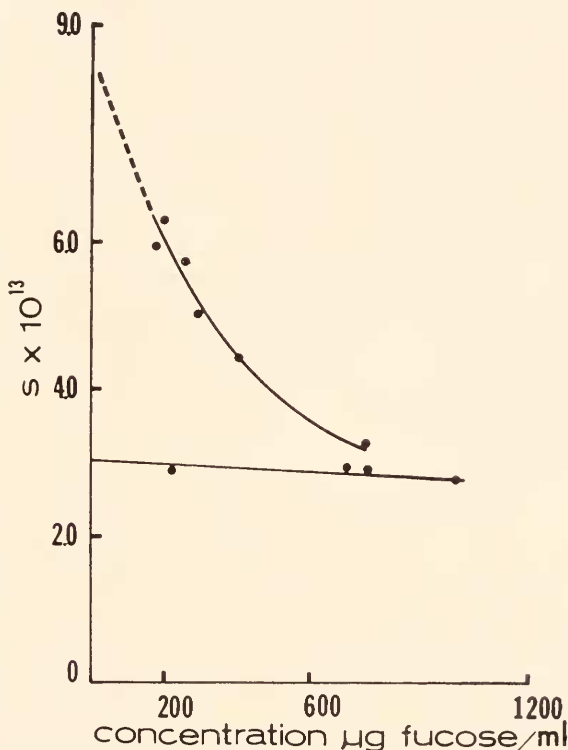


FIGURE 4a. Sedimentation velocity *vs.* concentration for multivalent and hydrogen peroxide formed univalent fertilizin. Upper curve: Multivalent fertilizin. Lower curve: Univalent fertilizin.

The viscosity change was followed with three different samples of fertilizin. Data from one of these are given in Figure 2. The rate of change is seen to be dependent on the $H_2O_2$ concentration. Following the completion of the experiments the samples were dialyzed against 0.1 *M* NaCl for 10 hours at 4° C. After dialysis the treated samples were found to be electrophoretically indistinguishable from univalent fertilizin formed by 2% $H_2O_2$ treatment for 30 minutes.

Similar experiments were conducted with fertilizins of *Tripneustes esculentus, Echinometra lucunter, Eucidaris tribuloides,* and *A. punctulata.* In all cases a drop in relative viscosity occurred in the presence of hydrogen peroxide, whereas the viscosity of control preparations containing no hydrogen peroxide showed no viscosity change even after 100 minutes at 30° C.

*Sedimentation velocity determinations*

Concentrations and $s_{20,w}$ values for multivalent and hydrogen peroxide formed univalent fertilizins are given in Table I. Photographs were taken at 16-minute intervals, the first exposure being made when speed was attained. A typical sedimentation pattern is illustrated in Figure 3.

The variation of the sedimentation coefficient of multivalent fertilizin with decreasing concentration (Fig. 4a, b) shows the behavior expected for a highly asymmetric molecule; the univalent material behaves as a more symmetric system.

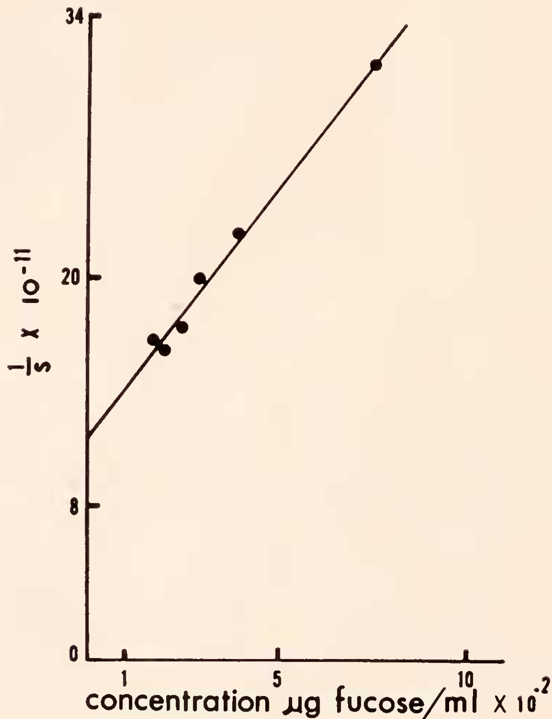


FIGURE 4b. 1/s *vs.* concentration for multivalent fertilizin.

With decreasing concentration the univalent peak tends to spread, suggesting heterogeneity (Tanford, 1961). The empirical sedimentation velocity behavior of asymmetric particles with concentration is given by the formula

$$1/s = 1/s^{\circ} + kc \text{ (Tanford, 1961).}$$

A plot of 1/s *vs.* c is a more reliable method of obtaining s° for multivalent fertilizin than the plot of s *vs.* c (Fig. 4b). Extrapolation to infinite dilution gives a value of 8.60 Svedberg for multivalent *Lytechinus* fertilizin and 3.05 Svedberg for univalent fertilizin formed by hydrogen peroxide treatment (Fig. 4a).

*Electrophoresis*

Freshly prepared multivalent fertilizin failed to migrate from the origin under the electrophoretic conditions employed. Univalent fertilizin formed by treatment with hydrogen peroxide is resolved into four bands, as determined by metachromatic staining (at least 50 trials). When multivalent fertilizin is retained at room temperature for several hours a spontaneous breakdown of the material is noted from its electrophoretic behavior. With prolonged storage at 37° C., *e.g.* 150 hours, no material remains at the origin on the cellulose acetate strips and the

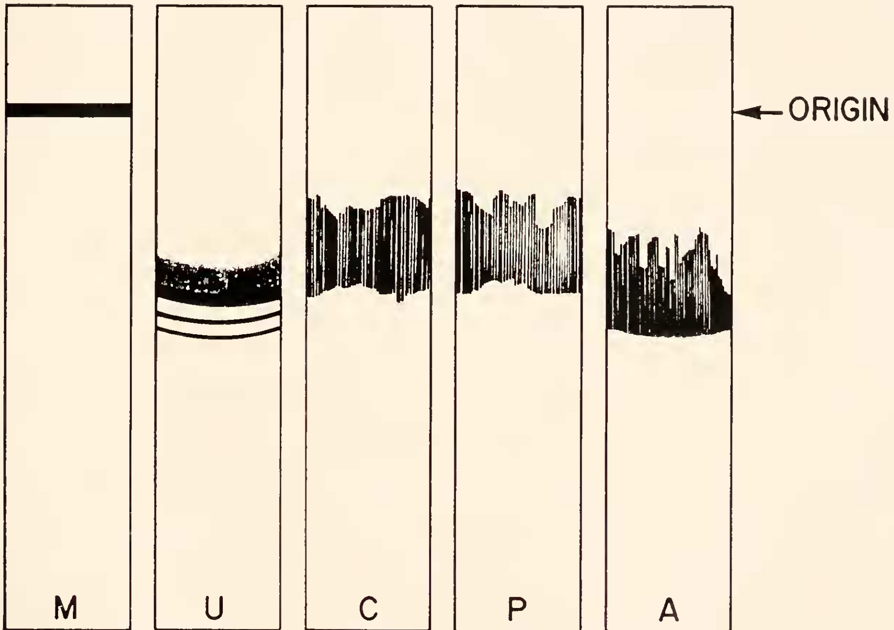


FIGURE 5. Cellulose acetate electrophoretic patterns of *Lytechinus* fertilizins. The solvent was borate buffer, pH 8.6 $\Gamma/2 = 0.05$. Two hundred volts were applied for 25 minutes. M: Multivalent fertilizin. U: Univalent fertilizin formed with hydrogen peroxide. C: Univalent fertilizin formed by treatment with chymotrypsin for 30 minutes at 30° C. P: Univalent fertilizin formed by treatment with pronase for 30 minutes at 30° C. A: Autodegraded univalent fertilizin formed by storing multivalent fertilizin at 37° for 50 hours.

material is functionally totally univalent. This univalent material formed by spontaneous breakdown of the multivalent form produces a diffuse streak rather than discrete bands in cellulose acetate electrophoresis, indicating that it is more heterogeneous than the univalent material formed by hydrogen peroxide. The rate of migration of the fastest moving fraction never exceeds that of the fastest moving component of the hydrogen peroxide formed univalent material. The spontaneous breakdown of the fertilizin may be due to the presence of digestive enzymes which were not removed during the washings of the eggs. Figure 5 shows the typical behavior of the different types of univalent fertilizin compared to the multivalent form.

The effects of the proteolytic enzymes, chymotrypsin and pronase, on the viscosity, electrophoretic behavior, and sperm-agglutinating properties were studied with three different samples of multivalent fertilizin. The addition of trypsin to a solution of multivalent fertilizin resulted in a precipitation. The addition of 300 $\mu g$. of pronase to a 5 ml. solution of multivalent fertilizin of relative viscosity 3.73 resulted in a decrease of the viscosity to 1.94 within one minute after the addition of the enzyme. At the end of that time the material was also univalent, as determined by sperm agglutination tests. Chymotrypsin-treated material was univalent on the basis of sperm agglutination 30 minutes after the addition of the enzyme. Figure 5 gives the electrophoretic patterns obtained with samples of fertilizin rendered univalent by treatment witht chymotrypsin and with pronase for 30 minutes. The electrophoretic behavior of the treated samples suggests that the enzymes acted on the original material in a degradative manner.

It seemed of interest to determine the effect of $H_2O_2$ on "spontaneously" formed univalent fertilizin since this produces a broad streak rather than discrete bands in electrophoresis. Two samples of spontaneously formed univalent fertilizin were treated with 2% hydrogen peroxide. The samples were univalent in terms of sperm agglutination and contained only migrating electrophoretic material. Two samples that were multivalent in terms of sperm agglutination, but which did contain some migrating material (partially degraded), were also treated. In all four cases the resultant univalent material produced the four metachromatic bands characteristic of the $H_2O_2$ formed univalent material.

These observations, and the course of viscosity change, suggest that intermediate forms may occur during hydrogen peroxide degradation of fertilizin. It was further suggested that these intermediates may be very similar in electrophoretic behavior to spontaneously formed univalent fertilizin.

The demonstration of such an intermediate(s) was carried out by arresting the action of $H_2O_2$ at intervals by addition of catalase to the fertilizin-hydrogen peroxide mixture. Two samples of multivalent fertilizin were treated with 2% hydrogen peroxide. At intervals, 4-ml. aliquots were removed and 5 $\mu g$. (5 $\lambda$) of the enzyme were added to the aliquot. A rapid evolution of gas, presumably oxygen, occurred when the catalase was added. Gas evolution lasted for several minutes. The last aliquot was removed at 120 minutes. At the end of that time 5-$\lambda$ samples of each aliquot were applied to cellulose acetate strips and electrophoresis was carried out in barbital buffer, pH 8.6. Alteration in electrophoretic behavior was rapid.

At the end of 10 minutes of treatment no material could be observed at the origin. The migrating material behaved as predicted. A broad migrating band was observed following a 1-minute treatment with $H_2O_2$. At the end of 20 minutes of treatment four metachromatic bands were observed. No change was noted over the next 100 minutes of reaction time.

One of the two samples was also treated with a lower concentration of hydrogen peroxide. Five milliliters of sample was treated with 0.6% hydrogen peroxide. Aliquots of 0.5 ml. were removed at intervals of 0, 5, 10, 20, 30, 60, and 135 minutes after the addition of the peroxide, and 1 $\mu g$. of catalase was added. As expected the breakdown of the material was much slower in this case. At the end of the 30 minutes some non-migrating material was still demonstrable; at 60 minutes all

material migrated; but breakdown identical to the normal univalent pattern was not noted until 135 minutes.

*Amino acid analysis*

Four samples each of multivalent and the hydrogen peroxide formed univalent fertilizins were examined for amino acid composition. At least 16 amino acids were found in each of the hydrolysates. In all runs two peaks appeared on the 50-cm. column after phenylalanine, and before lysine on the 10-cm. column. These peaks have been tentatively identified as glucosamine and galactosamine on the basis of their $R_f$ values compared to published values (Walborg *et al.,* 1963) and to standard amino acid-hexosamine (as hydrochlorides) mixtures. The $R_f$ values of the two peaks are identical with the $R_f$ values of the standard sugars on both the 50-cm. and 10-cm. columns. Quantitative calculations were based on values obtained for standards on the 10-cm. column. A high degree of similarity was noted between the amino acid compositions of the univalent and parent multivalent form. There were, however, differences in some amino acids. These differences were not uniform in all of the analyses. Further analyses are required in order to make a definitive statement concerning the amino acid compositions of multivalent and univalent fertilizins.

Free amino acids were found in all four multivalent fertilizin samples hydrolyzed with pronase.

*Protein staining*

Protein staining was attempted with Amido Schwarz and Ponceau S on cellulose acetate strips. No detectable protein-staining material was found in preparations of either multivalent or hydrogen peroxide formed univalent fertilizins. Protein tests on the cellulose acetate strips were done with different multivalent fertilizin solutions varying in concentration from 0.05 mg./ml. to 2.0 mg./ml., and with univalent fertilizin solutions over the concentration range of 0.05 mg./ml. to 3.0 mg./ml. Ten $\lambda$ of each solution were applied to the strips.

*Nitrogen*

Samples for nitrogen determination were dried by lyophilization and weighed. Kjeldahl determinations were then made on the dried material. Determinations on eight different samples of multivalent fertilizin yielded a value of 2.37% nitrogen, with a range of 2.12% to 2.87%.

*Amino sugars*

A total of three univalent and eight multivalent samples of known weight were hydrolyzed under conditions generally employed for amino sugars. The data are given in Table II. Maximal release of glucosamine and galactosamine seems to occur between 5 and 10 hours. The total amount of amino sugars found is of the order of 1% of the dry weight of the fertilizin.

One sample each of *Tripneustes* and *Echinometra* fertilizins was also found to contain peaks corresponding to the amino sugar peaks.

TABLE II

*Amino sugar content of Lytechinus fertilizins. Gram Residues per 100 grams material*

| Sample | Hours of hydrolysis | Glucosamine | Galactosamine |
|---|---|---|---|
| 11/16 M | 1 | 0.22 | 0.15 |
| 1/24 M | 1 | 0.19 | 0.15 |
| 11/24 M | 2 | 0.32 | 0.27 |
| 11/24 M | 2 | 0.33 | 0.35 |
| 2/7 M | 2 | 0.31 | 0.23 |
| 2/7 U | 2 | 0.38 | 0.23 |
| 9/1 M | 5 | 0.68 | 0.37 |
| 9/1 M | 10 | 0.46 | 0.25 |
| 11/16 M | 10 | 0.80 | 0.34 |
| 11/24 U | 10 | 0.53 | 0.36 |
| 11/18 U | 10 | 0.59 | 0.31 |

*Carbohydrate analysis*

Fucose was the only carbohydrate found by chromatography in hydrolysates of *Lytechinus* fertilizin. No non-methyl pentose was detected by the method of Dische and Shettles (1948).

TABLE III

*Fucose analyses of Lytechinus fertilizins*

A. Comparison of samples after dialysis

| Sample | Concentration $\mu$g. fucose/ml. | |
|---|---|---|
| | Multivalent | Univalent |
| 6/30/64 | 23.9 | 25.8 |
| 6/18/64 | 34.4 | 30.6 |
| 5/12/65 | 190.0 | 189.0 |
| 5/16/65 | 218.0 | 224.0 |
| 5/17/65 | 180.0 | 149.0 |
| 6/8/65 | 171.0 | 180.0 |

B. Fucose content of samples of known weight

| Sample | % Fucose | |
|---|---|---|
| | Multivalent | Univalent |
| 6/13/64 | 26.8 | 27.2 |
| 10/1/65 (1) | 26.9 | 20.5 |
| 10/2/65 | 24.3 | 23.3 |
| 10/1/65 (2) | 22.8 | 22.8 |
| 10/8/65 | 30.4 | 33.8 |
| 10/7/65 | 28.9 | 24.6 |
| Average | 26.7 | 25.5 |

Comparison of the fucose content of multivalent fertilizin and univalent fertilizin formed by hydrogen peroxide treatment was made on dried samples and samples obtained immediately after dialysis. Data comparing the fucose content of univalent and multivalent fertilizins are given in Table III. The fucose content of the two materials is identical.

## Discussion

These experiments demonstrate that the formation of univalent fertilizin is associated with a breakdown of the multivalent form. The different methods of preparing univalent fertilizin yield products which are physiologically equal in terms of action on sperm, but are different in physical behavior.

The existence of different physical forms of univalent fertilizin has been demonstrated by comparison of the electrophoretic behavior of univalent fertilizins formed by the action of hydrogen peroxide, proteolytic enzymes, and autodegradation. Of the three univalent forms studied, the most completely degraded, in terms of relative viscosity and electrophoretic behavior, is the one formed by the action of hydrogen peroxide. The observation that autodegraded univalent fertilizin can be further degraded to a form which is electrophoretically equivalent to the univalent fertilizin formed with hydrogen peroxide, suggests that the linkages cleaved during autodegradation may be the same or very similar to those initially attacked by hydrogen peroxide. This is further substantiated by the fact that during the hydrogen peroxide degradation of the multivalent fertilizin, it is also possible to demonstrate the existence of a form similar to the autodegraded form.

The formation of a univalent fertilizin by treatment with hydrogen peroxide has been shown to be dependent at least on the concentration of the peroxide (Fig. 2), for *Lytechinus* fertilizin. The degradation reaction does not proceed instantaneously for any of the five species tested. Hydrogen peroxide is known to degrade polysaccharides (see Moody, 1964 for review) but the mode of action is not clear. Metz (1942) obtained univalent fertilizins from *A. punctulata* and *S. purpuratus* by subjecting multivalent fertilizins to ionizing radiation. It is not clear whether the formation of univalent fertilizins in this case was due directly to the radiation or a secondary formation of peroxides from water (Moody, 1964).

The $s^{\circ}_{20,w}$ values of 8.60 and 3.05 obtained for multivalent, and hydrogen peroxide formed univalent, *Lytechinus* fertilizins, respectively, demonstrate that depolymerization has occurred in transition from the multivalent to the univalent form. Krauss (1949) suggested that univalent fertilizin is a depolymerization of multivalent fertilizin on the basis of the mucin clot reaction. Tyler (1949, 1956) and Runnström *et al.* (1942) obtained sedimentation velocity values for various multivalent sea urchin fertilizins. Unfortunately neither worker obtained an infinite dilution sedimentation constant. They were, however, able to conclude that the multivalent fertilizins of *P. miliaris* and *A. punctulata* behave as asymmetric particles in the ultracentrifuge. In the present work this was also found for the multivalent fertilizin of *L. variegatus*.

The chemical analyses reported here were done mainly to compare multivalent fertilizin and univalent fertilizin formed by hydrogen peroxide treatment. The glucosamine and galactosamine found in *Lytechinus* fertilizin are the first clear demonstration of such amino sugars in any fertilizin. Previous determinations of

amino sugars as components of fertilizins (see Vasseur, 1952) were not clear because of the interference of amino acids and carbohydrates in the test procedure.

The experiments of Hathaway and Metz (1961) indirectly suggest that univalent fertilizin may be composed of several fractions, not all of which combine with sperm. The electrophoretic behavior of univalent *Lytechinus* fertilizin formed with hydrogen peroxide shows that the material is composed of at least four metachromatic fractions. Stern and Metz (unpublished data) have been able to remove some of these fractions with homologous sperm. Since in no case were all of the fractions removed it is suggested that some portion(s) of the parent multivalent fertilizin are inactive in terms of sperm agglutination.

The structural relationship of the univalent fertilizin fractions to the parent multivalent form is at present not known. A chromatographic investigation similar to that conducted by Porter (1959, 1960) on rabbit antibodies might prove of interest in interpreting the structure of multivalent fertilizin.

## Summary

1. Treatment of multivalent *Lytechinus* fertilizins with hydrogen peroxide, proteolytic enzymes, or elevated temperatures results in the formation of univalent fertilizin.

2. Electrophoretic behavior showed that univalent fertilizins formed by the different means listed above are not physico-chemically equivalent. During the course of hydrogen peroxide action, forms intermediate to the initial material and final product are realized. Hydrogen peroxide formed univalent material is composed of at least four fractions as demonstrated by metachromatic staining on cellulose acetate. This suggests that degradation of fertilizin by hydrogen peroxide is nonrandom.

3. The main carbohydrate found in hydrolysates of *Lytechinus* fertilizins was the methyl pentose, fucose. The fucose content of multivalent and hydrogen peroxide formed univalent fertilizins was found to be identical following dialysis. Glucosamine and galactosamine were found to be components of *Lytechinus* fertilizin.

4. On the basis of sedimentation velocities, electrophoretic behavior, and lowered relative viscosity it is concluded that univalent fertilizin is a fragmentation product of multivalent fertilizin.

## LITERATURE CITED

Block, R. J., E. L. Durrum and G. Zweig, 1958. A Manual of Paper Chromatography and Paper Electrophoresis. New York: Academic Press.

Dische, Z., and L. B. Shettles, 1948. A specific color reaction of methyl pentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem.*, **175**: 595–603.

Fischer, R. B., 1961. Quantitative Chemical Analysis. Philadelphia: W. B. Saunders Co.

Formulae and Methods IV of the Marine Biological Laboratory Chemical Room, 1956. G. M. Cavanaugh, Editor. Woods Hole, Mass.: Marine Biological Laboratory.

Gray, H. E., and G. Fraenkel, 1954. The carbohydrate components of honeydew. *Physiol. Zoöl.*, **27**: 56–65.

Hathaway, R. R., and C. B. Metz, 1961. Interaction between *Arbacia* sperm and $S^{35}$-labelled fertilizin. *Biol. Bull.*, **120**: 360–369.

International Critical Tables of Numerical Data, Physics, Chemistry, and Technology, 1926. Vol. 5. E. W. Washburn, Editor in Chief. New York: McGraw Hill.

KRAUSS, M., 1949. A mucin clot reaction with sea urchin fertilizin. *Biol. Bull.*, **96**: 74–89.
LANGE, N. A., 1956. Handbook of Chemistry. New York: McGraw-Hill.
LILLIE, F. R., 1913. The mechanism of fertilization. *Science*, **38**: 524–528.
METZ, C. B., 1942. The inactivation of fertilizin and its conversion to the "univalent" form by x-rays and ultra violet light. *Biol. Bull.*, **82**: 446–454.
METZ, C. B., 1954. The effect of some protein group reagents on the sperm agglutinating action of *Arbacia* fertilizin. *Anat. Record*, **120**: 713–714.
METZ, C. B., 1957. Specific egg and sperm substances and activation of the egg. *In*: The Beginnings of Embryonic Development, A. Tyler, R. C. von Borstel, and C. B. Metz (eds.). American Association for the Advancement of Science, Washington, D. C., pp. 23–69.
MONROY, A., 1965. Chemistry and Physiology of Fertilization. New York: Holt, Rinehart and Winston.
MOODY, G. J., 1964. The action of hydrogen peroxide on carbohydrates and related compounds. *In*: M. L. Wolfrom (ed.) Advances in Carbohydrate Chemistry. Vol. 19, New York: Academic Press. pp. 149–180.
PORTER, R. R., 1959. The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. *Biochem. J.*, **73**: 119–126.
PORTER, R. R., 1960. Active fragments of antibody. Brookhaven Symposia in Biology. Number **13**: 203–209.
RUNNSTRÖM, J., A. TISELIUS AND E. VASSEUR, 1942. Zur Kenntnis der Gammonwirkungen bei *Psammechinus miliaris* und *Echinocardium cordatum*. *Arkiv Kemi*, **15 A**, No. 16: 1–18.
SCHACHMAN, H. K., 1959. Ultracentrifugation in Biochemistry. New York: Academic Press.
STEINER, R. F., 1965. The Chemical Foundations of Molecular Biology. Princeton: D. Von Nostrand.
TANFORD, C., 1961. Physical Chemistry of Macromolecules. New York: Wiley and Sons.
TYLER, A., 1940. Agglutination of sea urchin eggs by means of a substance extracted from the eggs. *Proc. Nat. Acad. Sci.*, **26**: 249–256.
TYLER, A., 1941. The role of fertilizin in the fertilization of the eggs of the sea-urchin and other animals. *Biol. Bull.*, **81**: 190–204.
TYLER, A., 1942. Specific interacting substances of eggs and sperm. *Western J. Surg. Obs. Gyn.*, **50**: 126–138.
TYLER, A., 1948. Fertilization and immunity. *Physiol. Revs.*, **28**: 180–219.
TYLER, A., 1949. Properties of fertilizin and related substances of eggs and sperm of marine animals. *Amer. Naturalist*, **83**: 195–219.
TYLER, A., 1956. Physico-chemical properties of the fertilizins of the sea-urchin, *Arbacia punctulata* and the sand-dollar *Echinarachnius parma*. *Exp. Cell Res.*, **10**: 377–386.
TYLER, A., AND S. W. FOX, 1940. Evidence for the protein nature of the sperm agglutinins of the keyhole limpet and the sea-urchin. *Biol. Bull.*, **79**: 153–165.
VASSEUR, E., 1948. Chemical studies on the jelly coat of the sea-urchin egg. *Acta Chem. Scand.*, **2**: 900–913.
VASSEUR, E., 1951. *Strongylocentrotus pallidus* (G. O. Sars) and *S. droebachiensis* (O. F. Müller) distinguished by means of sperm agglutination with egg-water and ordinary morphological character. Acta Borelia, A. Scientic. No. 2: 1–16.
VASSEUR, E., 1952. The Chemistry and Physiology of the Jelly Coat of the Sea Urchin Egg. Kihlstromstryck: A. B. Stockholm.
WALBORG, E. F., JR., B. F. COBB, III, M. ADAMS-MAYNE AND D. N. WARD, 1963. Semiautomatic analysis of glucosamine and galactosamine in protein hydrolysates. *Anal. Biochem.*, **6**: 367–373.